Improvement of Erythropoietin N-glycan Branching and Sialylation by Overexpression of Glycosyltransferases

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Abstract

The emergence of recombinant therapeutic proteins whose indications range from anti-cancer to rheumatoid arthritis treatments represented a significant revolution for modern pharmaceutical industry. When introduced into patients, the immunogenicity, bioactivity, and serum half-life of the therapeutic proteins are greatly impacted by their glycosylation patterns, especially the antennarity of the N-glycan and the level of terminal sialylation. To introduce α2,6-sialic acids and elevate overall sialylation level, a human α2,6-sialytransferase (ST6GAL1) was expressed in Chinese hamster ovary (CHO-K1) cells which produce recombinant human erythropoietin (EPO). In addition, both UDP-N-acetylglucosamine: α-1,3-D-mannoside β1,4-N-acetylglucosaminyltransferase (GNTIV/Mgat4) and UDP-N-acetylglucosamine:α-1,6-D-mannoside β1,6-N-acetylglucosaminyltransferase (GNTV/Mgat5) were further incorporated into the CHO-K1 cell line expressing EPO and ST6GAL1.

Firstly, stable clones of CHO-K1 cells expressing glycosyltransferases were established. CHO-K1 cell line expressing recombinant EPO (ChEPO) was transfected with ST6GAL1. The stable pool was created by drug selection and stable clone (ChEPO-S) was picked by lectin blot. Similarly, GNTIV and GNTV were simultaneously transfected into ChEPO-S cells. Once the stable pool was constructed, the stable clone (ChEPO-SG) was selected using western blot and lectin blot analysis.

The second goal of this thesis was to examine the expression and function of ST6GAL1, GNTIV and GNTV in ChEPO-S and ChEPO-SG cell lines. The expression of the transfected genes at both transcription and translation levels were confirmed by RT-

PCR and western blot, respectively. The impact of these glycosyltransferases on the level of terminal sialylation as well as the degree of branching of intracellular proteins and purified EPO from the various CHO cell lines was evaluated. Lectin blot analysis indicated that transfection of ST6GAL1 indeed introduced α2,6-sialic acids and increased overall sialylation level. Higher extent of β1,4 and β1,6 branching at the N-glycan trimannosyl core was also observed. HPLC analysis quantified the sialic acid content of recombinant human EPO purified from each cell line. Results demonstrated that recombinant human EPO produced by ChEPO-S and ChEPO-SG cells respectively contained ~26% and ~45% more sialic acids compared to EPO from ChEPO cells.

In this study, for the first time three glycosyltransferases, ST6GAL1, GNTIV and GNTV, were overexpressed in CHO-K1 cells in a simultaneous and coordinated manner. This strategy allows robust enhancement of the N-glycan complexity and terminal sialylation of recombinant therapeutic proteins produced by CHO cells.

Advisor: Dr. Michael Betenbaugh

Reader: Dr. Kevin Yarema

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Chapter 1: Introduction

1.1 Protein Glycosylation and Sialylation

Glycosylation of newly synthesized proteins is the most ubiquitous and common post-translational modification, which produces attachment of oligosaccharides to either asparagine (N-linked) or serine/threonine (O-linked) through covalent linkages [1, 2]. According to the SwissProt protein database, more than 50% of eukaryotic proteins are glycoproteins, with 90% of them are expected to contain N-glycosylation [3]. N-glycosylation is critical for glycoproteins of therapeutic importance, as it modulates the pharmacokinetics, immunogenicity, bioactivity, solubility, stability, protein folding and cellular processing [2, 4, 5].

N-glycans arise when sugar residues are added to the nascent protein in the endoplasmic reticulum (ER), and are further trimmed and processed in Golgi complex as the glycoproteins mature [6]. All N-glycans contain a saccharide core unit, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr (See Figure 1) [7].

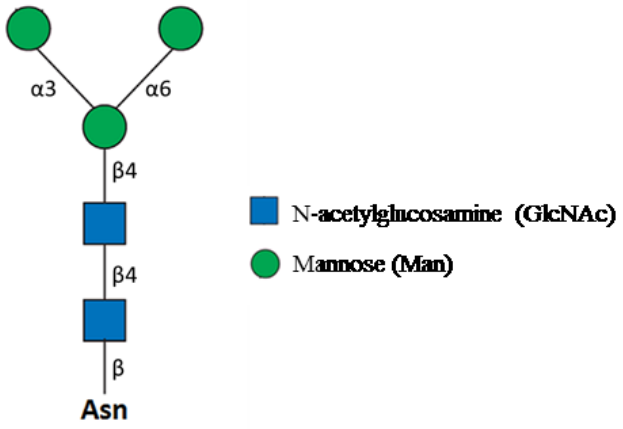


Figure 1. Pentasaccharide core of N-glycans. A conservative set of five-saccharide core unit, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr, can be found in all N-glycans.

1.1.1 Complex N-glycan Branching

The structures of N-glycans can be categorized into three types: (1) oligomannose, (2) complex, and (3) hybrid. The oligomannose type only contains mannose residues that are attached to the core structure (See Figure 2a). In the complex type, N-acetylglucosaminyltransferases (GlcNAcTs) originate two to four antennae from the α1,3 and α1,6 mannose residues of the core. A typical antenna in complex N-glycans consists of a galactose (Gal) β1,4-linked to a β1,2-GlcNAc, followed by a terminal N-acetylneuraminic acid (NeuNAc), or sialic acid (See Figure 2b). Finally, the hybrid type features characteristics of both oligomannose and complex type N-glycans, in which only mannose residues are connected to the Manα1–6 arm of the core, and one or two antennae are initiated from the Manα1–3 arm (See Figure 2c) [7, 8].

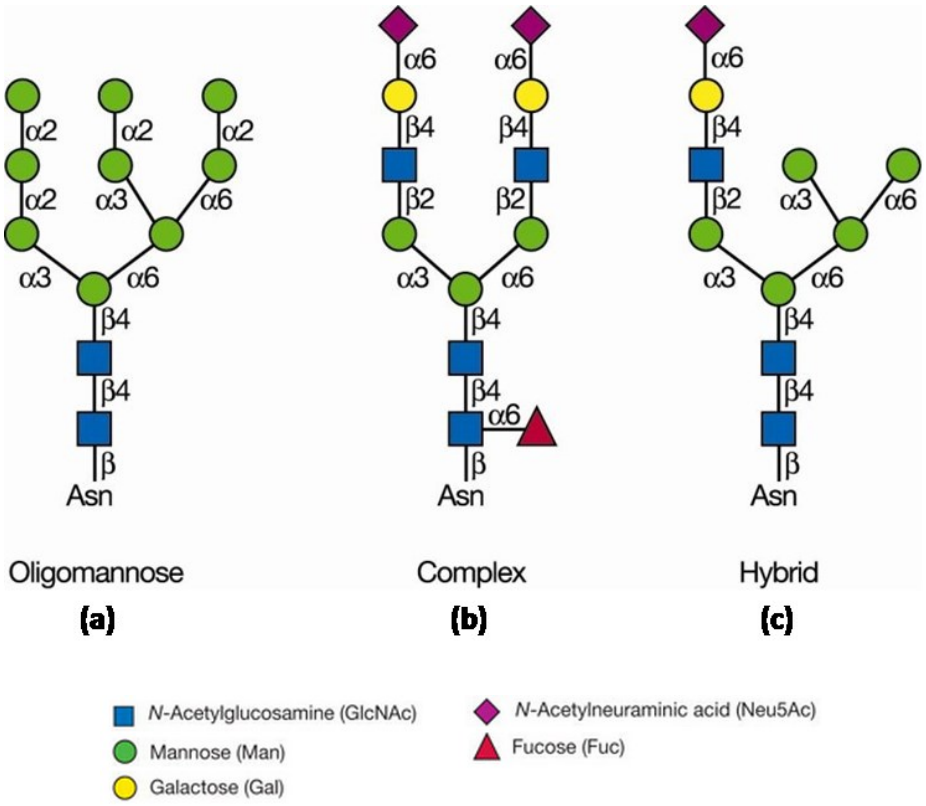


Figure 2. Three main structure types of N-glycans. (a) Oligomannose (b) Complex (c) Hybrid [7].

N-glycans exhibit complex-type structures in mammalian cells and the biosynthesis process involves a complicated glycosylation network of enzymes, including glycosidases and glycosyltransferases. An example N-glycosylation of a glycoprotein expressed in Chinese Hamster Ovary (CHO) cells is represented in Figure 3. Once the glycoprotein is translocated into the Golgi apparatus and trimmed by Golgi α-mannosidases (α MAN-I and α MAN-II), it is modified by a variety of N-acetylglucosaminyltransferases (GNTI to GNTVI or Mgat1 to Mgat 5) [9, 10]. GNTI and GNTII attach GlcNAc residues onto each the α1,3 and α1,6 mannose branch, creating the classic complex biantennary structure. Meanwhile, the N-glycan structure can be further remolded by additional N-acetylglucosaminyltransferases to form more complex structures. In particular, GNTIV (UDP-N-acetylglycosamine:α-1,3-D-mannoside β-1,4-N-acetylglcosaminyltransferase, EC 2.4.1.145) and GNTV (UDP-N-acetylglycosamine:α-1,6-D-mannoside β-1,6-N-acetylglcosaminyltransferase, EC 2.4.1.155) are in charge of generating tri-antennary and tetra-antennary structures.

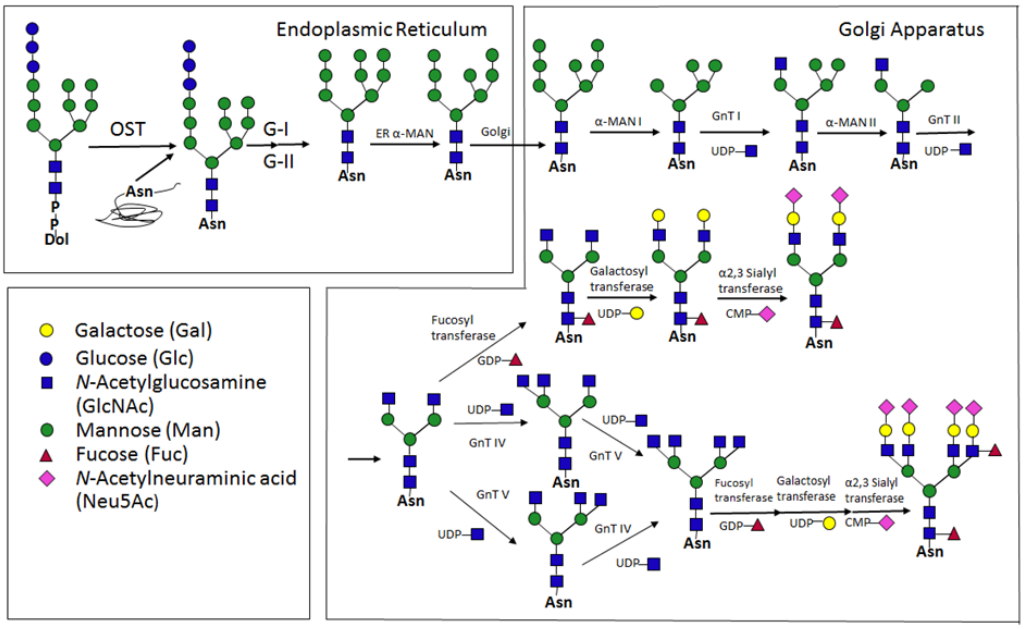


Figure 3. N-glycosylation pathway for a therapeutic protein produced in CHO cell [11].

The complex N-glycan branched structures regulate many important biological functions, such as cancer metastasis, T-cell activation and autoimmunity [12, 13]. The N-glycan branching of a glycoprotein affects not only the molecular weight of the protein, but also the clearance rate of the protein by glomeruli of kidneys [14]. Furthermore, the number of N-glycan branches directly decides the amount of sites available for further N-glycosylation processing to create various glycoforms. In particular, a galactose (Gal) residue can be added to the GlcNAc residues, and subsequently the sugar chain can be elongated by addition of GlcNAc residues to form lactosamine repeats (Galβ1,4-GlcNAc) [15]. On the other hand, a sialic acid residue can also be transferred to the terminal positions of galactosylated oligosaccharide chains [16, 17].

1.1.2 Sialylation

In mammalian cells, sialic acids capped N-glycans are constituents of various glycoproteins of biological importance, such as lectins, antibodies (e.g. IgG) and luteinizing hormones [18]. Sialic acid is a large family of negatively charged acidic 9-carbon monosaccharide, with N-acetylneuraminic acid (NeuNAc or NANA) as the most common form [19]. Masking the terminal Gal of the N-glycan chain to avoid endocytosis-mediated capture by receptors of the hepatocytes, sialic acids prevent the fast removal of the glycoprotein from circulation [20]. Previous studies have proven that hypersialylation indeed enhances the overall efficacy and circulatory half-life of many important therapeutic glycoproteins, including but not limited to asparaginase, leptin and erythropoietin [21-25].

For the occurrence of sialylation, a cytidine monophospho-sialic acid (CMP-SA) is formed in the nucleus by the conversion of sialic acid by CMP-SA synthetase,

transported to the Golgi apparatus by a CMP-SA transporter, and attached to the acceptor oligosaccharide by the sialyltransferase (See Figure 4).

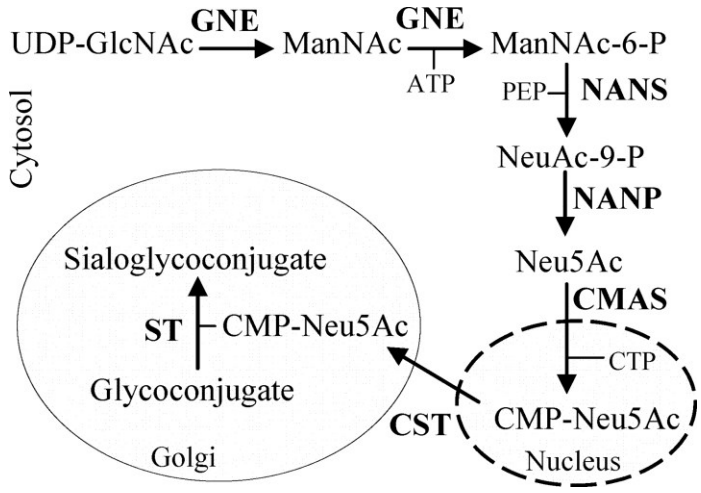


Figure 4. Mammalian biosynthesis pathway for CMP-sialic acid. The enzymes involved in the process are indicated: GNE, NANS, NANP, CMAS, CMP-Neu5Ac transporter (CST), and sialyltransferase (ST) [26].

To maximize the level of sialylation of a glycoprotein in mammalian cells, two opposing processes need to be considered. Firstly, a sialic acid can be converted to a CMP-SA substrate and then added to the terminal N-glycan by the sialyltransferase. Meanwhile, a terminal sialic acid can also be removed by sialidase cleavage [11]. There are two major families of sialytransferases in mammalian cells: ST3GAL and ST6GAL families. Specifically, ST6GAL1 from the ST6GAL family transfers the sialic acid to the terminal Gal, creating an α-2,6 linkage, whereas ST3GAL3, ST3GAL4, and ST3GAL6 from the ST3GAL family create α-2,3 linked sialylation [27].

1.2 Importance of Glycosylation and Sialylation of Recombinant Proteins Produced in Chinese Hamster Ovary Cells

Nowadays, intensive academic and industrial research focus on biopharmaceutical proteins such as monoclonal antibodies, growth factors, hormones, cytokines, and fusion proteins [28]. These proteins have a broad range of indications from anti-cancer to rheumatoid arthritis treatments, showing great clinical and economic importance. Over 60% of the biopharmaceutical proteins are glycoproteins, and they can undergo a series host-generated responses after taken by a patient [29]. For example, if the glycoprotein is orally administered, it can get denatured by the acid in the gastrointestinal tract. If it is parenterally administered, it can be recognized and removed by renal and hepatic enzymes. In addition, a glycoprotein can be considered as antigenic, and thus can trigger the stimulation of an immune response [18]. Hence, glycoengineering of biopharmaceutical proteins with reduced antigenicity and improved efficacy is of great need and importance.

Currently, the majority of biopharmaceutical glycoproteins is produced in mammalian cell expression systems [30]. Although other expression systems, such as yeast, insect and plants, have also been utilized to produce recombinant proteins, mammalian expression platform is principally chosen mainly due to its capability to produce glycosylation patterns similar to human [31]. Mammalian cells frequently used in biopharmaceutical industry include Chinese hamster ovary (CHO), baby hamster kidney (BHK-21), murine myeloma (NS0 and Sp2/0) and human cells [11]. Among them, about 70% of recombinant therapeutic proteins are produced in CHO cells because they can grow in suspension and chemically defined medium, reach a high-density culture, be

easily transfected, perform post-translational modifications including glycosylation, and normally do not cause immunogenic responses or safety issues compared to murine myeloma and human cells [11, 32, 33]. However, glycosylation patterns found in CHO cells have discrepancies compared to those in human cells, especially at the level of terminal sialylation. For example, N-glycans on native proteins expressed in human cells contain a mixture of both α2,3- and α2,6-linked sialic acids, but recombinant glycoproteins produced in most CHO cells usually exclusively display α2,3-linked sialylation due to their lack of the expression of α2,6-sialytransferases [34-36]. Moreover, on N-glycans of glycoproteins expressed in CHO cells, a type of non-human epitope, N-glycolnylneuraminic acid (Neu5Gc or NGNA), can be found (See Figure 5). As a derivative of Neu5Ac, Neu5Gc is a major sialic acid that can be found in a variety of mammalian cells. However, human cells do not contain Neu5Gc because they lack CMP-Neu5Ac hydroxylases which catalyze the conversion of Neu5Ac into Neu5Gc [37]. Therefore, the existence of Neu5Gc on therapeutic glycoproteins can potentially lead to immunogenic responses and compromised efficacies in patients [38]. The amount of Neu5Gc varies from protein to protein. For instance, fetuin contains about 7% of Neu5Gc of total sialic acids and causes immunogenic responses. On the contrary, there is only a low level of Neu5Gc (around 1% of total sialic acids) present on recombinant erythropoietin (EPO), so immunogenic responses activated are negligible [39].

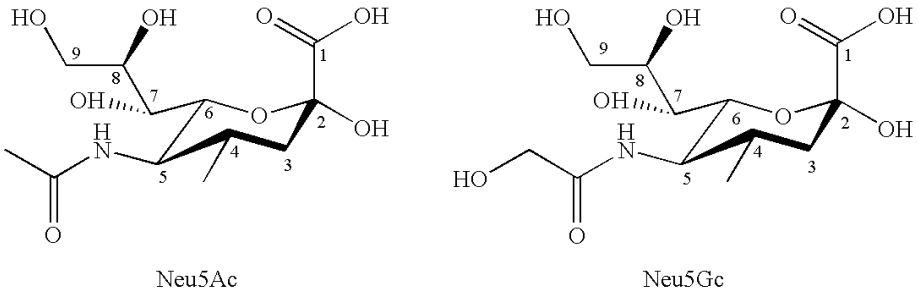


Figure 5. Two types of sialic acids commonly seen on glycoproteins produced in mammalian cells. N-acetylneuraminic acid (Neu5Ac) (left). N-glycolnylneuraminic acid (Neu5Gc) (right).

1.3 Erythropoietin as a Model Protein for Glycoengineering Study

As previously mentioned, the branches of the complex N-glycan and terminal sialic acids affect the stability, bioactivity and efficacy of therapeutic glycoproteins. Thus, it is desirable to engineer the CHO cell line in order to produce recombinant glycoproteins with more N-glycan branches and higher level of sialylation. Erythropoietin (EPO) serves as a model glycoprotein for this glycoengineering study because it is heavily glycosylated and it exhibits considerable molecular heterogeneity.

Human EPO is a hormone mainly synthesized in the kidney that functions to regulate red blood cell maturation and production [40]. Recombinant human EPO is an important therapeutic protein for the treatment of anemia associated with severe renal damage, HIV infection, and cancer chemotherapy [41, 42]. Consisting of a polypeptide of 165 amino acids and a high content of glycans, human EPO has an overall molecular weight of 34 – 39 kDa [42]. The glycoprotein contains 3 N-glycosylation sites (Asn 24, Asn 38, and Asn 83) and 1 O-glycosylation site (Serine 126) [43]. The glycan chains comprise about 40% of the molecular weight of human EPO and mostly cover the surface of the protein [44]. Each of the three N-glycan chains of human EPO has the potential for

different branching ranging from 2 to 4, and each branch contains a potential terminal sialic acid [44].

Besides EPO, several other glycoproteins, such as human interferon- γ (INF- γ) and acetylcholinesterase (AChE), have also been utilized as model proteins for glycoengineering studies.

Chapter 2: Incorporation of ST6GAL1, GNTIV and GNTV into CHO-K1 Cells

2.1 Enhancing the Glycosylation and Sialylation of Recombinant Glycoproteins

As mentioned in Chapter 1, it is highly desirable to improve the extent of glycosylation, especially at the level of terminal sialylation, of recombinant therapeutic glycoproteins produced by CHO cells, because it can enhance the stability, bioactivity and efficacy of the proteins. Many approaches can be utilized to influence the level of glycosylation, including alteration of the activity of glycosyltransferases for sugar residue transfer, the action of glycosidases on the glycan degradation, and the amount of nucleotide sugar substrate [30]. This glycoengineering study focuses on increasing the availability and activity of glycosyltransferases in order to improve the N-glycan structures.

2.1.1 Overexpression of Sialylation Pathway Enzyme

Due to the particular importance of sialic acids on therapeutic glycoproteins, it is beneficial to create hypersialylation by engineering the sialytransferases. As noted previously, unlike human glycoproteins that contain a mixture of both α2,3- and α2,6-linked sialylation, recombinant glycoproteins produced by CHO cells bear sialic acids with exclusively α2,3-linkages. Therefore, overexpression of heterologous α2,6-sialyltransferase becomes a conducive method. Bragonzi et al. successfully established a CHO cell line as a universal host for glycoprotein production by stably expressing the α2,6-sialyltransferase gene. They observed that the transgene was stably integrated into the CHO cell genome, and the recombinant sialylatransferase was correctly localized in the Golgi. The transfected CHO cell line showed similar growth characteristics compared

to the wild type CHO cells. In addition, they expressed IFN-γ in their host CHO cell line, and found that the purified IFN-γ carried 40.4% α2,6- and 59.6% α2,3-linked sialic acid residues, and that the glycoprotein exhibited enhanced pharmacokinetics in clearance studies [45]. Jassal et al. transfected a rat α2,6-sialyltransferase gene into the CHO-K1 cell line expressing IgG3 antibody, and observed an enhanced overall sialylation level of IgG-Fc-linked carbohydrate. The ratio of α2,6- to α2,3- sialic acid also increased from 0.0:1.0 to 0.9:1.0 [46]. Several other studies have also shown that overexpression of α2,6-sialytransferase into CHO cells introduced the α2,6-linked sialylation, elevated the level of terminal sialic acids, and enhanced the bioactivity of the recombinant glycoprotein [47, 48].

2.1.2 Overexpression of N-acetylglucosaminyltransferases

Complex N-glycan may include bi-, tri-, and tetra-antennary structures. As mentioned in Chapter 1.1.1, GNTIV and GNTV are responsible for the tri- and tetra-antennary structures. Fukuta et al. transfected genes encoding GNTIV and GNTV enzymes into human IFN-γ producing CHO cells, and indeed increased the percentages of tri- and tetra-antennary structures of total N-glycans. Without transfection, IFN-γ produced by CHO cells mainly exhibited the classic biantennary structure. When GNTIV was overexpressed, triantennary N-glycan with β1,4 branch at the trimannosyl core increased up to 66.9% of the total sugar chains. Transfection of GNTV increased the triantennary N-glycan with β1,6 branch up to 55.7%. When both GNTIV and GNTV were overexpressed simultaneously, the tetraantennay N-glycosylation pattern was elevated to 56.2% [48].

2.2 Previous Work

Our study investigated the influence of several glycosyltransferases overexpressed in CHO-K1 cells on the level of sialylation and N-glycan branching of the recombinant human EPO. Due to the length of the study, some work has been done prior to this thesis. Previously, the ChEPO stable cell line has been established and the effects of each ST6GAL1, GNTIV and GNTV have been characterized. The recombinant human EPO was transfected into CHO-K1 cells, and by drug selection, the stable pool was constructed. The single clone with the highest EPO expression level was selected using indirect ELISA and additionally confirmed using western blot analysis. The stable clone was termed ChEPO and utilized as the host cell line for further glycoengineering experiments in our study.

In addition, each of ST6GAL1, GNTIV and GNTV has been transiently transfected into the ChEPO cell line in order to get some preliminary results on how the overexpression of these glycosyltransferases could affect the production of recombinant human EPO as well as the pattern of protein N-glycosylation. Results showed that co-expression of either ST6GAL1 or GNTIV with EPO did not influence the expression level of EPO. In contrast, when GNTV was transfected into ChEPO cells, a decreased production of EPO was observed. Using the lectin blot analysis, it was verified that overexpression of ST6GAL1 and GNTV significantly increased the level of α2,6-linked sialylation and β1,6-linked GlcNAc in both the total protein and the purified EPO produced by ChEPO cells.

Therefore, in this thesis, further glycoengineering work will be continued. Firstly, ST6GAL1 will get transfected into the ChEPO cell line, and stable pools will be selected

with drugs. Limiting dilution will be performed to generate single clones, and these single clones will be screened for EPO production as well as the respective N-glycosylation patterns using western blot and lectin blot analysis. The selected stable clone will be termed ChEPO-S. Next, ST6GAL1, GNTIV and GNTV will be simultaneously overexpressed in the ChEPO cell line, and the stable clone will be picked in a similar manner and named ChEPO-SG. With the establishment of the wild-type CHO-K1 (WT), ChEPO, ChEPO-S and ChEPO-SG cell lines, we will examine the expression of recombinant human EPO, ST6GAL1, GNTIV and GNTV in each cell line on both mRNA and protein level using RT-PCR and western blot analysis, respectively. Following confirmation of the functions of transfected glycosyltransferases will be carried out by lectin blot analysis of total protein and purified EPO from each cell line. In addition, HPLC analysis will be conducted to quantify the sialic acid content of purified EPO produced in each CHO cell line.

2.3 Materials and Methods

2.3.1 Cell Lines

Adherent CHO-K1 cells (Thermo Fisher Scientific) were grown in 100mm TC-treated culture dishes (Corning) in F-12K medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). Cells were maintained at 37ºC and 5% CO_2 in a Series 8000 – Direct Heat and Water Jacket CO_2 incubator (Thermo Fisher Scientific). Every two to four days, when cells reached about 70 – 80% confluent, they were subcultured at a ratio between 1:10 and 1:40 using 0.25% Trypsin-EDTA (Life Technologies).

2.3.2 Transfection

The ST6GAL1, GNTIV and GNTV constructs were transfected into EPO producing CHO-K1 cells (ChEPO) using Lipofectamine 2000 reagent (Life Technologies) in OptiMEM Reduced Serum Media (Life Technologies). ChEPO cells were seeded onto a 6-well clear TC-treated cell culture plate (Corning) at appropriate densities one day in advance of the transfection. The ChEPO cells were then incubated in the OptiMEM medium with Lipofectamine 2000 reagent and plasmid DNA complex for 24 hours. Transfected stable pools were acquired by drug selection using blasticidin (EPO-S) and zeocin (EPO-SG).

2.3.3 Isolation of Single Clones by Limiting Dilution

ChEPO cells transfected with ST6GAL1, GNTIV and GNTV were stained with Trypan Blue (Sigma-Aldrich) and counted using a hemocytometer. The cells were subjected to serial dilutions to a concentration of 1000 cells/ml. Then single clones were established by further diluting and seeding 0.7 cell/well in 96-well clear TC-treated cell culture plates (Corning). 2ml of culture medium with appropriate concentrations of selection drugs were added to each well. After incubation for 5 to 7 days at 37^{o}C and 5% CO_2, plates were screened for single clones under the microscope. Two additional days later, plates were checked again to make sure no secondary colonies had grown. Several single clones were randomly picked, passaged to and expanded in 24-well clear TC-treated cell culture plates (Corning). Selection drugs were still added to maintain the transfected genes.

2.3.4 Reverse Transcription PCR

Total RNA was purified from each wild-type CHO-K1, ChEPO, ChEPO-S and ChEPO-SG cells using the RNeasy Mini Kit (Qiagen), according to the manufacturer protocols. To investigate the corresponding mRNA expression level of the transfected human EPO, ST6GAL1, GNTIV and GNTV genes, 1μg of total RNA from each cell line was taken and used as the template for RT-PCR. PCR primers for each gene are outlined in Table 1. The PCR products were further subjected to electrophoresis on a 1% agarose gel at 60V for 50min, and then visualized by ethidium bromide staining.

Gene	Primer Direction	Primer Sequence
EPO	Forward	5'-atgggcgtgcacgagtgtc-3'
	Reverse	5'- tctatcgccggtccggcaa-3'
ST6GAL1	Forward	5'- aaccttatccctaggctgca-3'
	Reverse	5'-ggtgcagcttacgataagtc-3'
GNTIV	Forward	5'-gtaaggatgcgttgaataagtt-3'
	Reverse	5'- tttgctttgttctccatcttac-3'
GNTV	Forward	5'- gttgtttgttggacttgggtt-3'
	Reverse	5'- acttcagcatgtccttgtcc-3'

Table 1. The forward and reverse primers used in RT-PCR analysis for the mRNA expression of EPO, ST6GAL1, GNTIV and GNTV.

2.3.5 Protein Purification

The wild-type CHO-K1, ChEPO, ChEPO-S and ChEPO-SG cell lines were each cultured in 100mm culture dishes in F-12K medium supplemented with 10% FBS. To

obtain the total protein lysate, when cells reached 90% confluent, medium was removed, and cells were lysed with RIPA buffer followed by brief sonication.

The recombinant human EPO was purified from each cell line using the MagneHis protein purification system (Promega) according to the manufacturer's instruction for purification of polyhistidine-tagged, secreted proteins from cultured mammalian cells. The only modification made in this study was that 40µl of MagneHis Ni-particles were added to 1ml of culture supernatant.

2.3.6 SDS-PAGE

Protein concentrations of purified EPO samples were measured using the BCA Assay (Thermo Scientific) prior to the SDS-PAGE. Aliquots of purified EPO were stained with 5x SDS sample buffer supplemented with 2-mercaptoethanol, and then denatured by boiling at 95°C for 5 minutes. Samples were loaded onto 10% Mini-PROTEAN polyacrylamide gels (BIO-RAD), and proteins were separated at a constant voltage of 180V for 45 minutes at room temperature.

2.3.7 Western Blot

After SDS-PAGE, proteins were transferred from the polyacrylamide gel to the Immun-Blot polyvinylidene difluoride (PVDF) membrane (BIO-RAD) by blotting for 60 minutes at a constant 100V. The PVDF membrane was treated prior to protein transfer by soaking it in 100% methanol, rinsing with deionized (DI) water for one minute, and finally submerging it in the transfer buffer.

For western blot, the membrane was blocked at room temperature with 5% milk in PBST (phosphate-buffered saline with 0.5% Tween 20) for an hour, and then incubated in primary antibodies diluted in 5% milk for another hour on a shaker (See

Table 2). The membrane was then washed three times by PBST, 20min each time, and incubated with HRP-conjugated secondary IgG antibody (Cell Signaling Technology) for an extra hour. The membrane was thoroughly washed with PBST for three times again.

Protein	Primary Antibody	Secondary Antibody
EPO	Mouse anti-EPO (Abcam)	Anti-mouse IgG
ST6GAL1	Mouse anti-ST6 (Sigma)	Anti-mouse IgG
GNTIV	Rabbit anti-Mgat4 (Abcam)	Anti-rabbit IgG
GNTV	Mouse anti-Mgat5 (Abcam)	Anti-mouse IgG

Table 2. Primary and secondary antibodies used in western blot analysis for EPO, ST6GAL1, GNTIV and GNTV.

Finally, the membrane was visualized with chemiluminescent detection of the secondary antibody using the Lumina Forte Western HRP Substrate (Milipore) in a Molecular Imager ChemiDoc XRS (BIO-RAD) with Quantity One Software (BIO-RAD). The densitometry analysis was performed using ImageJ software (NIH) according to the blot analysis protocol.

2.3.8 Lectin Blot

For lection blot, after proteins were transferred to the PVDF membranes as previously described (See Chapter 2.3.7), the membranes were blocked in 1× Carbo-Free solution (Vector Labs) for one hour at room temperature. Next, membranes were incubated for another one hour at room temperature in *Biotinylated Sambucus Nigra Lectin* (SNA), *Datura Stramonium Lectin* (DSL), or *Phaseolus Vulgaris Leucoagglutinin* (PHA-L) which were diluted by 1:5000 in 5 ml of TBST (Tris-buffered saline with 0.5%

Tween 20). Then the membrane was washed thoroughly with TBST and continuously incubated in *Avidin D, Horseradish Peroxidase* (Av-HRP) diluted by 1:10000 in 5 ml of TBST. The membranes were again washed with TBST to eliminate unspecific binding and developed as previously described (See Chapter 2.3.7).

2.4 Results and Discussion

2.4.1 Selection of the Stable Clone of ChEPO-S

ST6GAL1 (PubMed Gene ID: 6480) cDNA (OriGene) which encodes the α2,6-sialyltransferase was subcloned into pEF6/V5-his TOPO TA. The plasmid DNA was transfected into ChEPO cells. To obtain the ChEPO-S stable pool, selection drugs were added to the cell culture medium. The transfected ChEPO cells kept undergoing selection process until the wild-type ChEPO cells reached less than 10% cell viability. After the establishment of the ChEPO-S stable pool, the cells were harvested and lysed. Equal amount of total intracellular protein from seven ChEPO-S single clones were subjected to SDS-PAGE and lectin blotting with SNA which reacts to the α2,6-sialic acid linkage (See Figure 6). The single clone with the highest reaction activity with SNA (Lane 6) was selected as the ChEPO-S stable clone.

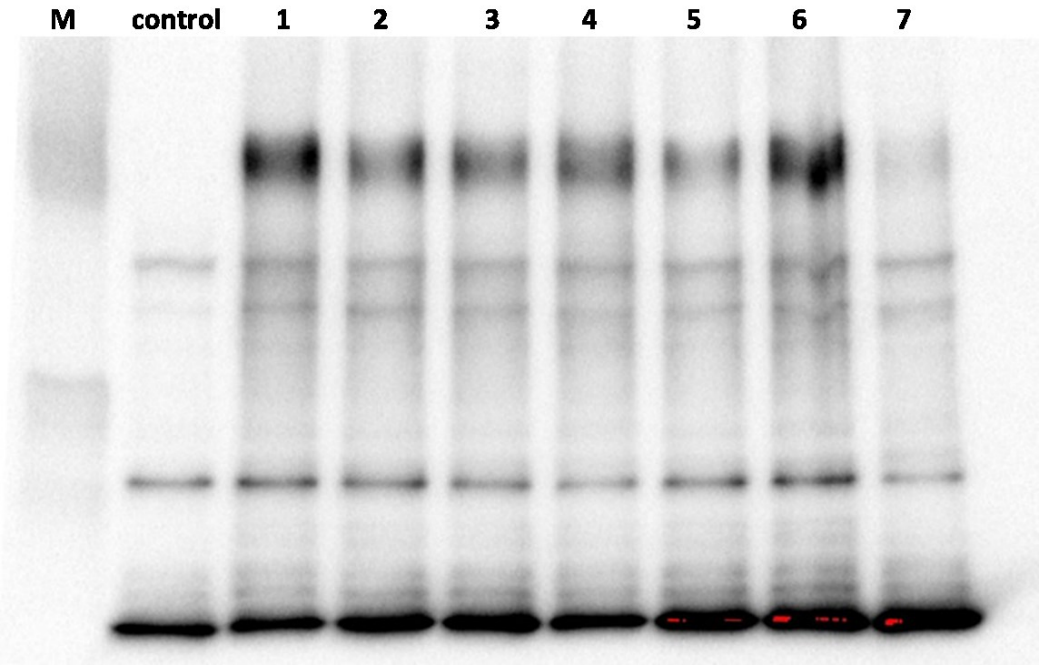


Figure 6. Lectin blot with SNA of total intracellular protein from seven ChEPO-S stable clones. M: Protein marker; control: total protein from ChEPO cells; 1-7: total intracellular protein from ChEPO-S stable clones.

2.4.2 Selection of the Stable Clone of ChEPO-SG

To create the ChEPO-SG cell line, human GNTIV and GNTV were additionally transfected into the ChEPO-S cell line. Because human GNTIV and GNTV genes are naturally present in human cells, they were cloned by reverse-transcription PCR (RT-PCR) from HEK 293 cells. Primer sequences used to capture GNTIV and GNTV are listed in Table 3.

Gene	Primer Direction	Primer Sequence
GNTIV	Forward	5’- atgaggctccgcaatggaact-3’
	Reverse	5’-tggtttctcagatgatcagttggtgg-3’
GNTV	Forward	5’-atggctctcttcactccgtgg-3’
	Reverse	5'-ctataggcagtctttgcagagagcc-3'

Table 3. Primers used in RT-PCR to clone GNTIV and GNTV from HEK 293 cells.

Similar to the selection process of ChEPO-S stable clone, human GNTIV and GNTV cDNA were inserted into pBudCE4.1 through the NotI/XhoI and SalI/Xbal sites, respectively, with GNTIV under EF1α promoter and GNTV under CMV promoter. The DNA construct was transfected into ChEPO-S cells. To obtain the ChEPO-SG stable pool, selection drugs were added to the cell culture medium. The transfected ChEPO cells kept undergoing selection process until the ChEPO-S cells reached less than 10% cell viability. Once the ChEPO-SG stable pool was created, 23 single clones were randomly picked for cell line selection. Total intracellular protein was prepared from each of the 23 ChEPO-SG clones, and subjected to SDS-PAGE and lectin blot with PHA-L. Of all the 23 clones, nine clones (Lane 3, 6, 7, 8, 9, 13, 14, 15 and 19) exhibited relatively higher binding activity with PHA-L, suggesting higher expression and function of GNTV (See Figure 7). These nine clones were picked and further analyzed for GNTIV and EPO expression.

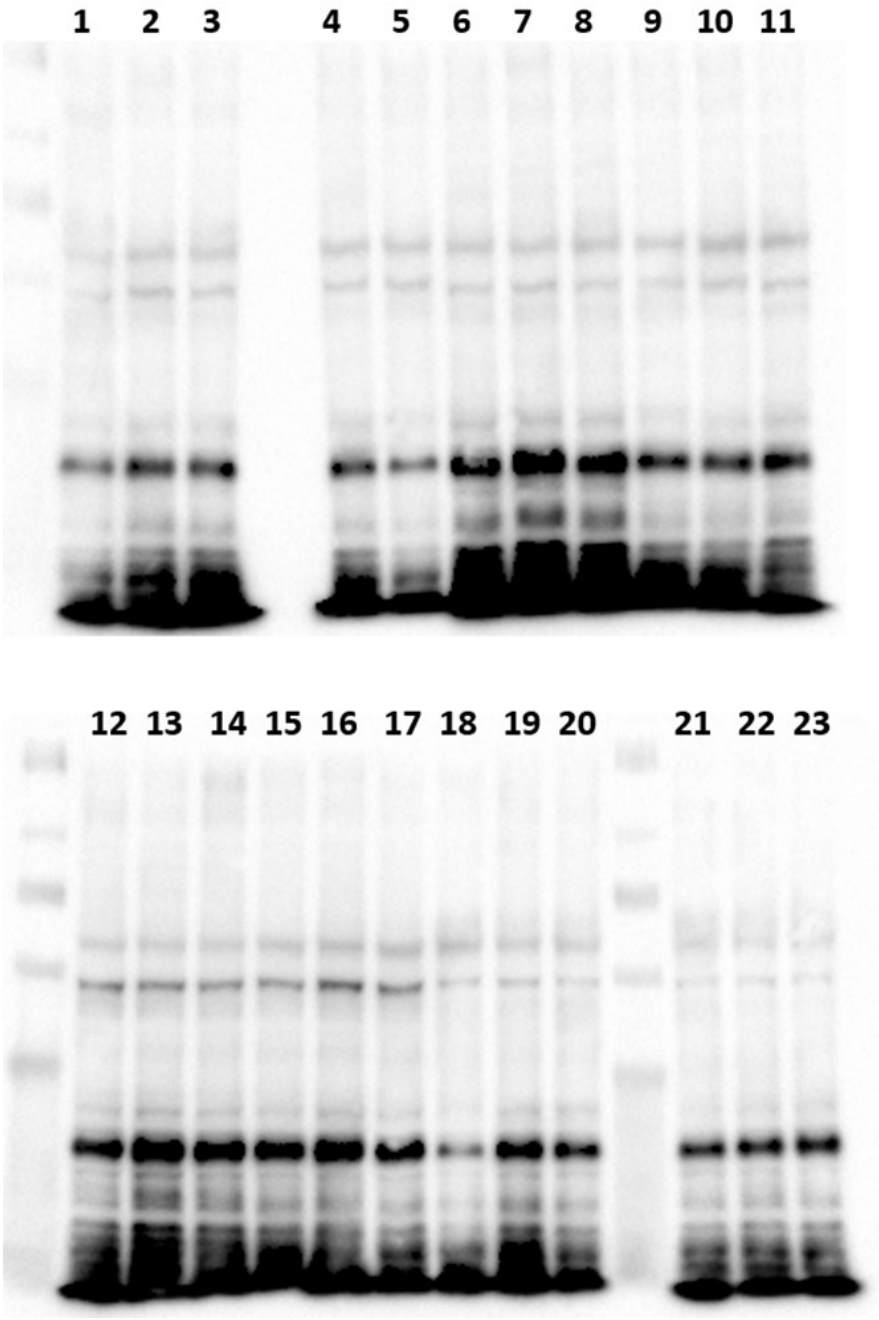


Figure 7. Lectin blot analysis for ChEPO-SG stable clones. Total intracellular protein was each prepared from 23 ChEPO-SG stable clones and analyzed by lectin blot with PHA-L to detect the level of β1,6 branch at the trimannosyl core of N-linked carbohydrate.

For the nine ChEPO-SG clones, GNTIV and EPO proteins were analyzed by SDS-PAGE and western blot. As the anti-GNTIV western blot depicted, five samples

(Lane 1, 2, 7, 8 and 9) had two bands, probably as a result of non-specific binding. The rest four samples (Lane 3, 4, 5 and 6) exhibited one single band, indicating the correct expression of GNTIV protein (Figure 8).

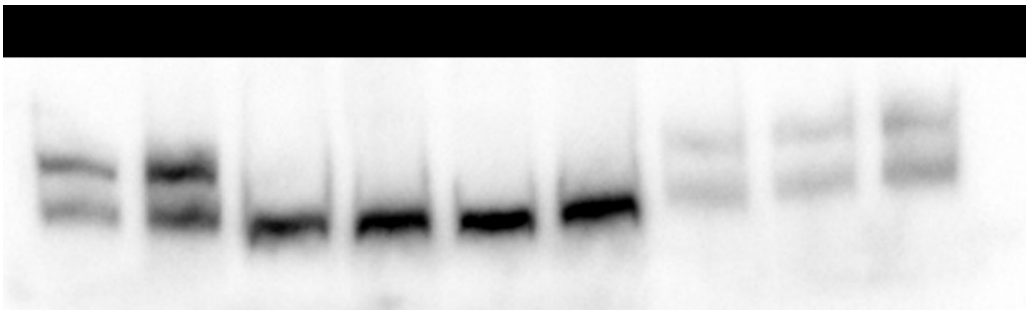


Figure 8. Anti-GNTIV western blot analysis for ChEPO-SG stable clones. GNTIV protein was purified from nine ChEPO-SG cell lines and analyzed by anti-GNTIV western blot.

On the other hand, as the anti-EPO western blot result illustrated, all nine clones successfully produced human EPO protein, but three of them (Lane 1, 4, 9) showed relatively higher expression (See Figure 9). Combined with the results of previous PHA-L lectin blot and anti-GNTIV western blot, Clone 4 was chosen as the stable ChEPO-SG cell line, as it demonstrated great GNTV function, correct GNTIV expression, and high EPO production.

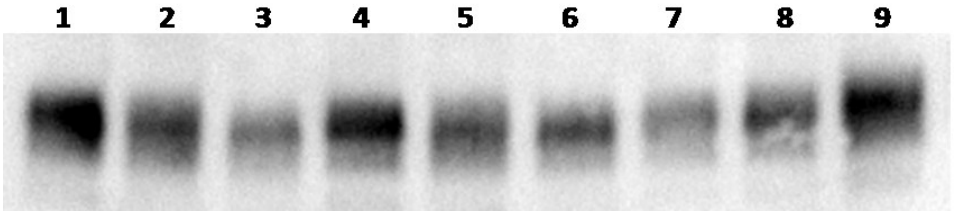


Figure 9. Anti-EPO western blot analysis for ChEPO-SG stable clones. Recombinant human EPO protein was purified from nine ChEPO-SG cell lines and analyzed by anti-EPO western blot.

2.4.3 Expression of ST6GAL1, GNTIV and GNTV on Transcription Level

In order to examine the expression of transfected human EPO, ST6GAL1, GNTIV and GNTV at the mRNA level, RT-PCR analysis of wild-type CHO-K1, ChEPO, ChEPO-S and ChEPO-SG cell lines was carried out (Figure 10). Because no heterologous gene was transfected, wild-type CHO-K1 cell line (Lane 1) had no EPO, ST6GAL1, GNTIV or GNTV expression at the transcription level. ChEPO (Lane 2) only contained EPO, while ChEPO-S (Lane 3) contained both EPO and ST6GAL1 transcripts. Transfected with all EPO, ST6GAL1, GNTIV and GNTV genes, ChEPO-SG cell line (Lane 4) indeed showed all four corresponding mRNAs. In the RT-PCR analysis, actin was used as an internal control.

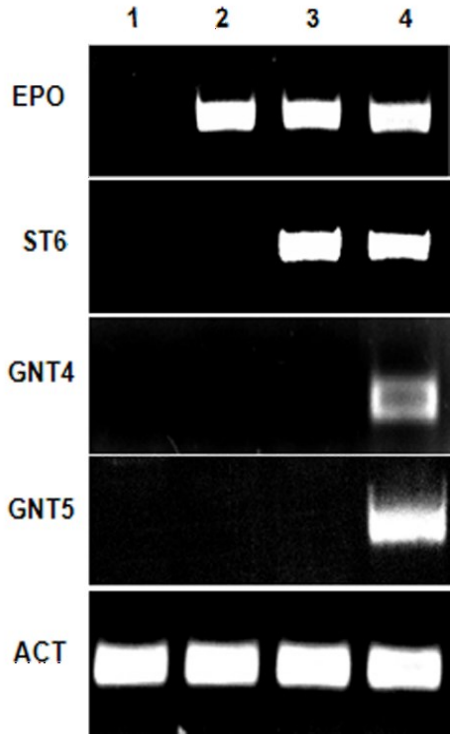
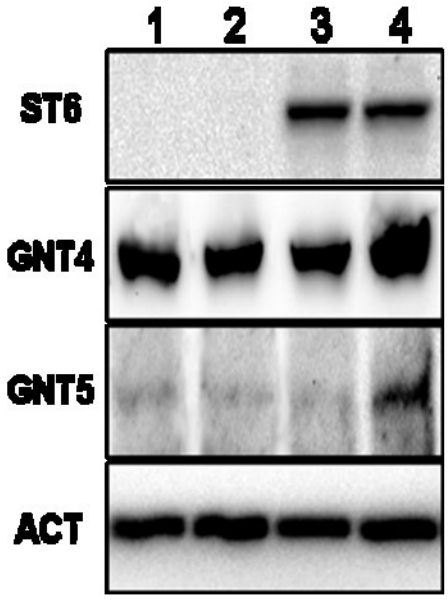


Figure 10. RT-PCR analysis for genes transfected into various CHO-k1 cell lines. Lane 1: Wild-type CHO-K1 cell (WT); Lane 2: ChEPO; Lane 3: ChEPO-S; Lane 4: ChEPO-SG.

2.4.4 Expression of ST6GAL1, GNTIV and GNTV on Translation Level

Since the mRNA expression of ST6GAL1, GNTIV and GNTV were confirmed using RT-PCR, the protein expression needs to be assessed as well. Total intracellular protein was prepared from each WT, ChEPO, ChEPO-S and ChEPO-SG cell lines. ST6GAL1, GNTIV and GNTV were then separated by SDS-PAGE and detected by corresponding antibodies in western blot analysis (See Figure 11). Consistent with the RT-PCR result, expression of ST6GAL1 protein was only found in ChEPO-S (Lane 3) and ChEPO-SG (Lane 4) cell lines. Although ChEPO-SG (Lane 4) showed obviously strongest reaction activities to anti-GNTIV and anti-GNTV antibodies, background binding can be seen in all cell lines. It is because endogenous GNTIV and GNTV from CHO cells display a significantly high amino acid similarity (~90%) to their human versions, and they also react to the antibodies used in western blot. Still, ChEPO-SG (Lane 4) exhibited 1.3 and 3.3 times higher level of GNTIV and GNTV enzymes compared to WT (Lane 1).



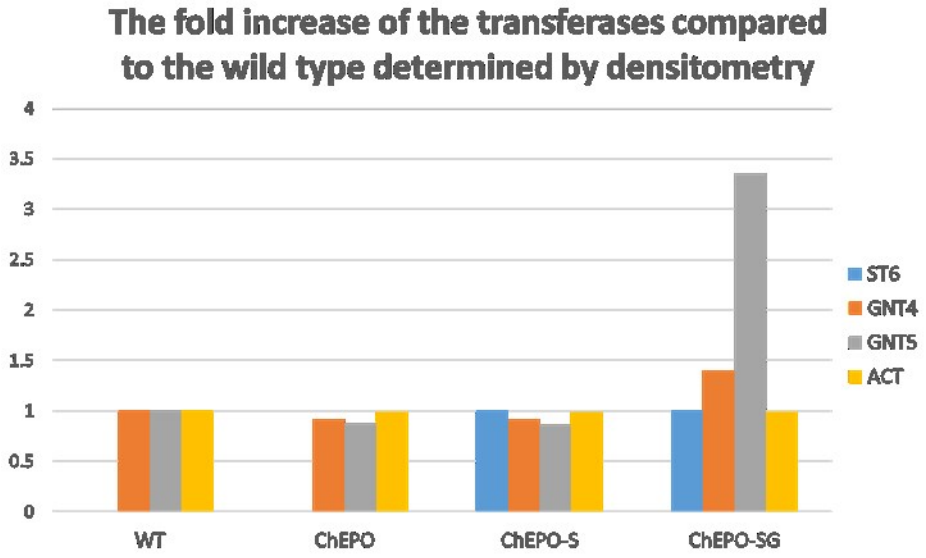


Figure 11. Western blot analysis for transfected human glycosyltransferases. (a) Lane 1: Wild-type CHO-K1 cells (WT); Lane 2: ChEPO; Lane 3: ChEPO-S; Lane 4: ChEPO-SG. (b) The fold increase of the ST6GAL1, GNTIV and GNTV proteins in ChEPO, ChEPO-S and ChEPO-SG cell lines compared to those in WT cell line. Note: Because there was zero expression of ST6GAL1 in WT and ChEPO, the fold increase in ChEPO-S and ChEPO-SG was considered as 1.

Chapter 3: Enhanced N-glycan Branching and Sialylation of Recombinant Human Erythropoietin

3.1 Glycoengineering for the Development of Biosimilars

With the development of biotechnology, recombinant therapeutic proteins have become an important medical option for a variety of indications. Recently, patents for a number of recombinant therapeutic protein products on market have expired or about to expire, such as interferons, human growth hormone, and erythropoietin. Therefore, alternatives of these recombinant proteins, biosimilars, have been widely studied and developed [49]. Unlike small-molecule generics, biosimilars are not identical to the reference products because recombinant therapeutic proteins are very complex and require multifaceted development and manufacturing processes [50].

In particular, glycosylation has an important role in the development of biosimilars for two reasons. Firstly, as mentioned previously in Chapter 1.2, glycosylation has a large influence on the stability, solubility, pharmacokinetics, bioactivity and immunogenicity of recombinant therapeutic proteins. Secondly, unlike DNA and proteins that can be synthesized based on gene or amino acid sequences, glycosylation is processed not according to template sequences but in a complex network involving a hundreds of enzymes and transporters, leading to variable glycan structures among different cell lines and even cell clones. Hence, glycoengineering is a critical part of the development of biosimilars in order to reproduce the glycosylation pattern of the reference products [51].

Nowadays, many biosimilar studies are focusing on the erythropoietin. Epoetin is recombinant human erythropoietin used to treat anemia in patients with chronic kidney

failure and cancer patients receiving chemotherapy. Epoetin is manufactured by Amgen, and this drug is marketed by Amgen under the trade name Epogen and by the subsidiary of Johnson & Johnson, Janssen Biotech, under the trade name Procrit. Currently, all biosimilar epoetin products in clinical trials have a highly similar amino acid sequence compared to the native human EPO, but they have different production cell lines, manufacturing processes, dosage regimens, routes of administration, and most importantly glycosylation patterns [52]. Thus, in spite of the same molecular mechanism of action, these products showed different pharmacologic and clinical properties from the innovator epoetin drug. Successful biosimilar epoetins must show a high level of glycosylation-based similarity to the reference product, Epogen. Some biosimilars currently licensed in Europe include Abseamed (Medice Arzneimitel Putter, Germany), Binocrit (Sandoz GmbH Kundl, Austria), and Epoetin alfa Hexal (Hexal Biotech, Germnay) [53].

Glycoengineering is essential for the development not only for the biosimilar epoetins, but also other biopharmaceuticals such as monoclonal antibodies. Although antibodies from the IgG class harbor only a single glycosylation site in the Fc region, and sometimes an additional site in the Fab region, the glycosylation is critical for the antibody-dependent cell-cytotoxicity (ADCC) and thus crucial for the efficacy of the drug [54]. Recently, a mogamulizumab with the trade name Poteligeo was approved in Japan as the first glycoengineered biosimilar antibody to reach the market [55]. Since glycosylation is of great importance for the development of biosimilars, it is beneficial to glycoengineer mammalian cell lines and thus create a novel and stable glycosylation profile.

3.2 Materials and Methods

3.2.1 Cell Lines

CHO-K1 cells were cultured as previously described in Chapter 2.3.1.

3.2.2 Immunoaffinity Purification of EPO

Before protein purification, wild-type CHO-K1, ChEPO, ChEPO-S and ChEPO-SG cell lines were each cultured in 100mm culture dishes in 8ml F-12K medium supplemented with 10% FBS. When cells reached 90% confluent, culture medium was changed to 8ml OptiMEM. In order to purify the secreted EPO, the cell culture supernatants were collected, filtered through membranes with a pore size of 0.45 μm, and then concentrated five times by centrifugal ultrafiltration using Amicon Ultra-15 Centrifugal Filters with a molecular weight cutoff (MWCO) of 10 kDa (Minipore).

The concentrated samples were loaded onto a column consisting of Ni-Nitrilotriacetic Acid (Ni-NTA) agarose matrix (Promega) to which His-tag has a high binding affinity. After loading, the column was thoroughly washed with PBS. The bound EPO was then eluted with PBS supplemented with 250 μM imidazole. The eluent fractions were immediately dialyzed with PBS and loaded to the Superdex 75 gel filtration column (GE life sciences). To evaluate the purity of purified EPO, the eluted samples were subjected to 10% SDS-PAGE followed by Coomassie Blue staining. Rest of the protein samples were stored at -20^{o}C.

3.2.3 SDS-PAGE

SDS-PAGE analysis was carried out as previously described in Chapter 2.3.6.

3.2.4 Western Blot

Western blot analysis was performed as previously described in Chapter 2.3.7.

3.2.5 Lectin Blot

Western blot analysis was conducted as previously described in Chapter 2.3.8.

3.2.6 Sialic Acid Analysis by High Performance Liquid Chromatography

The sialic acid content of purified EPO was measured by reverse-phase HPLC with fluorescence detection [56]. 400μl of 25mM sulfuric acid was added to 1μg purified EPO samples and the mixtures were heated at 80°C for 1h to hydrolyze the sialic acid. Then the samples were combined with 400μl of 7.0mM 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (MDB; Dojindo), and incubated at 60°C for 2.5h so that the sialic acid turned into highly fluorescent derivatives. N-acetylneuraminic acid (Neu5Ac) was diluted from 50 to 1000 μg/ml as a standard. Next, the MDB-labeled sialic acids from EPO were analyzed by HPLC (1260 Infinity; Agilent Technologies). A reverse-phase column with a length of 150mm and a particle size of 2.7 μm was used for separation (Poroshell120-ED-C18; Agilent Technologies). Sialic acids elution was monitored by a fluorescent detector (G1316A, Agilent) with the excitation and emission wavelengths at 367 nm and 445 nm.

3.3 Results and Discussion

3.3.1 Lectin Blot Analysis for Various Erythropoietin Producing Cell Lines

Previously, the expression of GNTIV and GNTV were confirmed at both the transcription and translation level (See Chapter 2.4.3 and 2.4.4). However, the functions of human GNTIV and GNTV in CHO cells still need to be evaluated. Therefore, lectin blot was performed on the total intracellular protein prepared from WT, ChEPO, ChEPO-S and ChEPO-SG cells lines. Biotinylated lectins were utilized to detect specific glycan structures: SNA reacts specifically to the α2,6 sialic acid, PHA-L binds to the β1,6 branch

at the trimannosyl core of N-linked carbohydrate (GlcNAcβ1-6 Manα1-6 Manβ-), and DSL reacts to the β1,4 branch at the trimannosyl core of N-linked carbohydrate [57].

ChEPO-S cell line showed a significantly higher amount of α2,6 sialic acid compared to WT and ChEPO cell lines, but ChEPO-SG had even higher content than ChEPO-S. Densitometry analysis of the lectin blot indicated that the α2,6-sialylation level in ChEPO-S and ChEPO-SG cell lines are 2.4 and 2.6 times higher than the WT cell line, respectively. In spite of the background binding of PHA-L and DSL in WT, ChEPO and ChEPO-S cell lines, ChEPO-SG exhibited a remarkably increased binding activities, and densitometry analysis showed that compared to WT cell line, ChEPO-SG had 1.9- and 4.6-fold elevation of β1,6- and β1,4- branched N-glycan levels (See Figure 12).

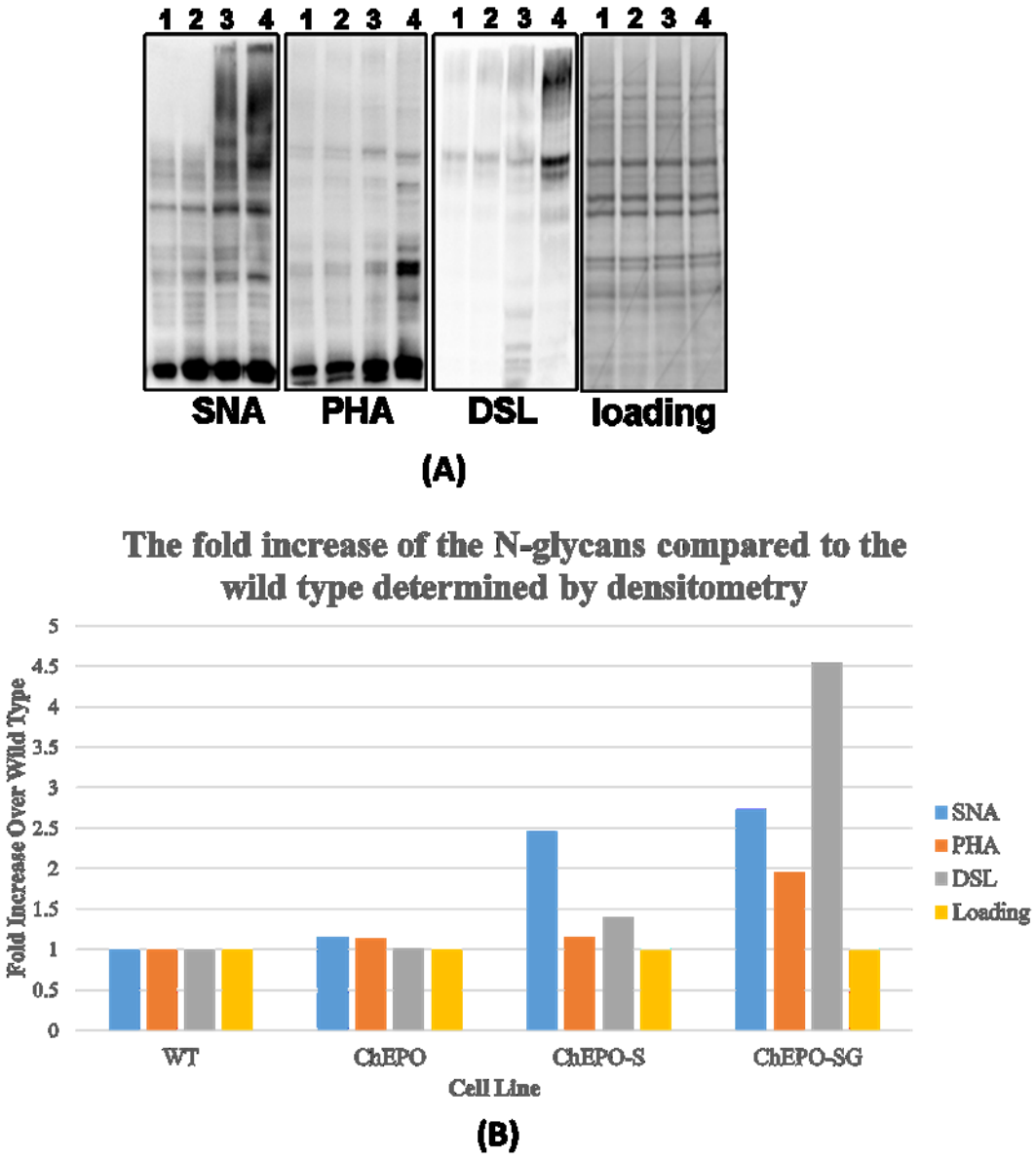


Figure 12. Lectin blot analysis for total intracellular protein from various CHO cell lines. (a) Total cellular protein was analyses by lectin blot with SNA (left), PHA (middle), DSL (right). 1: WT; 2: ChEPO; 3: ChEPO-S; 4: ChEPO-SG. Similar amount of total protein was loaded based on Coomassie blue staining. (b) The fold increase of the reaction activities to SNA, PHA and DSL of total proteins in ChEPO, ChEPO-S and ChEPO-SG cell lines compared to those in WT cell line.

3.3.2 Recombinant Erythropoietin Purified by Ni-NTA and Detected by Lectins

To investigate the impact of ST6Gal1, GNTIV and GNTV on the model glycoprotein EPO, the same amount of purified recombinant human EPO from each ChEPO, ChEPO-S and ChEPO-SG cell lines were subjected to SDS-PAGE and lectin

blot analysis. During the SDS-PAGE, recombinant EPO purified from ChEPO and ChEPO-S cell lines showed similar migration rates, but recombinant EPO from ChEPO-SG migrated much slower, which suggests that EPO produced in ChEPO-SG had a larger molecular weight compared to recombinant EPO from ChEPO and ChEPO-S cell lines. The increase of EPO size might be the result of the expansion of N-glycans due the expression of transferases, GNTIV and GNTV.

Next, lectin blot analysis with each SNA, PHA-L and DSL on recombinant human EPO was carried out. In contrast to recombinant human EPO produced in ChEPO cell line which showed no binding activity to SNA, EPO purified from ChEPO-S and ChEPO-SG had a significant binding reaction, proving a notable increase in α2,6-sialylation. Without transfection of GNTIV and GNTV, ChEPO-S cell line indeed showed similar binding activity to PHA-L and DSL with ChEPO. Recombinant EPO produced by the ChEPO-SG cell line revealed a 2.5- and 2.7-fold higher binding activity to PHA-L and DSL compared to EPO from ChEPO and ChEPO-S cells, indicating an increased amount of β1,4- and β1,6- branched glycans (See Figure 13).

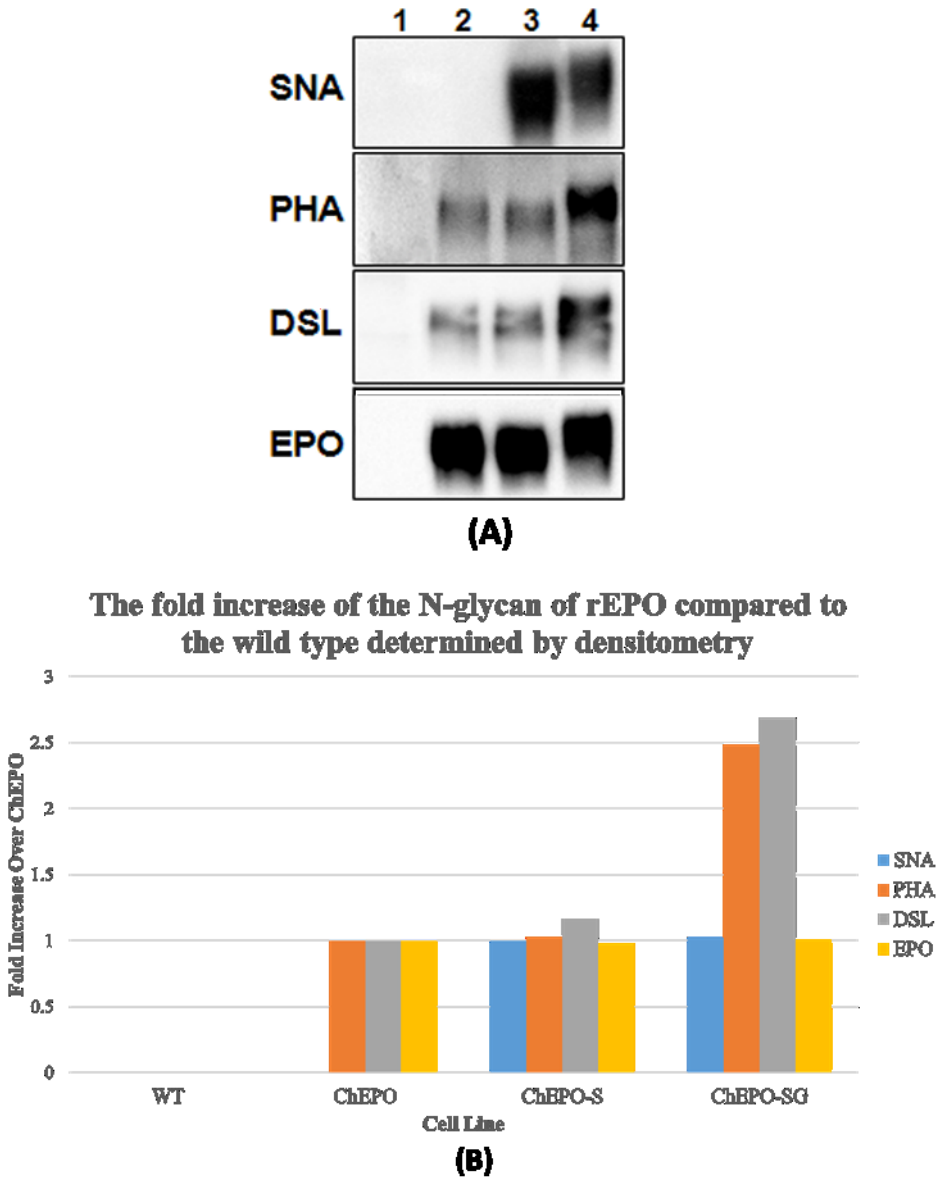


Figure 13. Lectin blot analysis of recombinant human EPO purified from various cell lines. (a) EPO was purified from each cell line with Ni-NTA affinity chromatography and detected by lectins: SNA, PHA and DSL. Western blot of similar amount of recombinant human EPO purified from each cell line was used as the control. 1: Wild-type CHO-K1 cells (WT); Lane 2: ChEPO; Lane 3: ChEPO-S; 4: ChEPO-SG. (b) The fold increase of reaction activities toward SNA, PHA and DSL of recombinant human EPO purified from ChEPO, ChEPO-S and ChEPO-SG compared to ChEPO cell line.

3.3.3 Sialic Acid Content of Recombinant Erythropoietin Determined by HPLC

In order to investigate the effects of transfected ST6GAL1, GNTIV and GNTV on the sialylation level of recombinant human EPO produced in CHO-K1 cells, sialic acids

were released from purified recombinant EPO, derivatized with the fluorescent MDB, and then separated on a reverse-phase C18 column. Serial concentrations of sialic acid standards were measured first to serve as a reference of peak time and sialic acid concentration. The recombinant human EPO sialic acid samples from each cell line were subjected to HPLC analysis. The main peak which showed at the same time as the standard represented the sialic acid content of the EPO sample (See Figure 14).

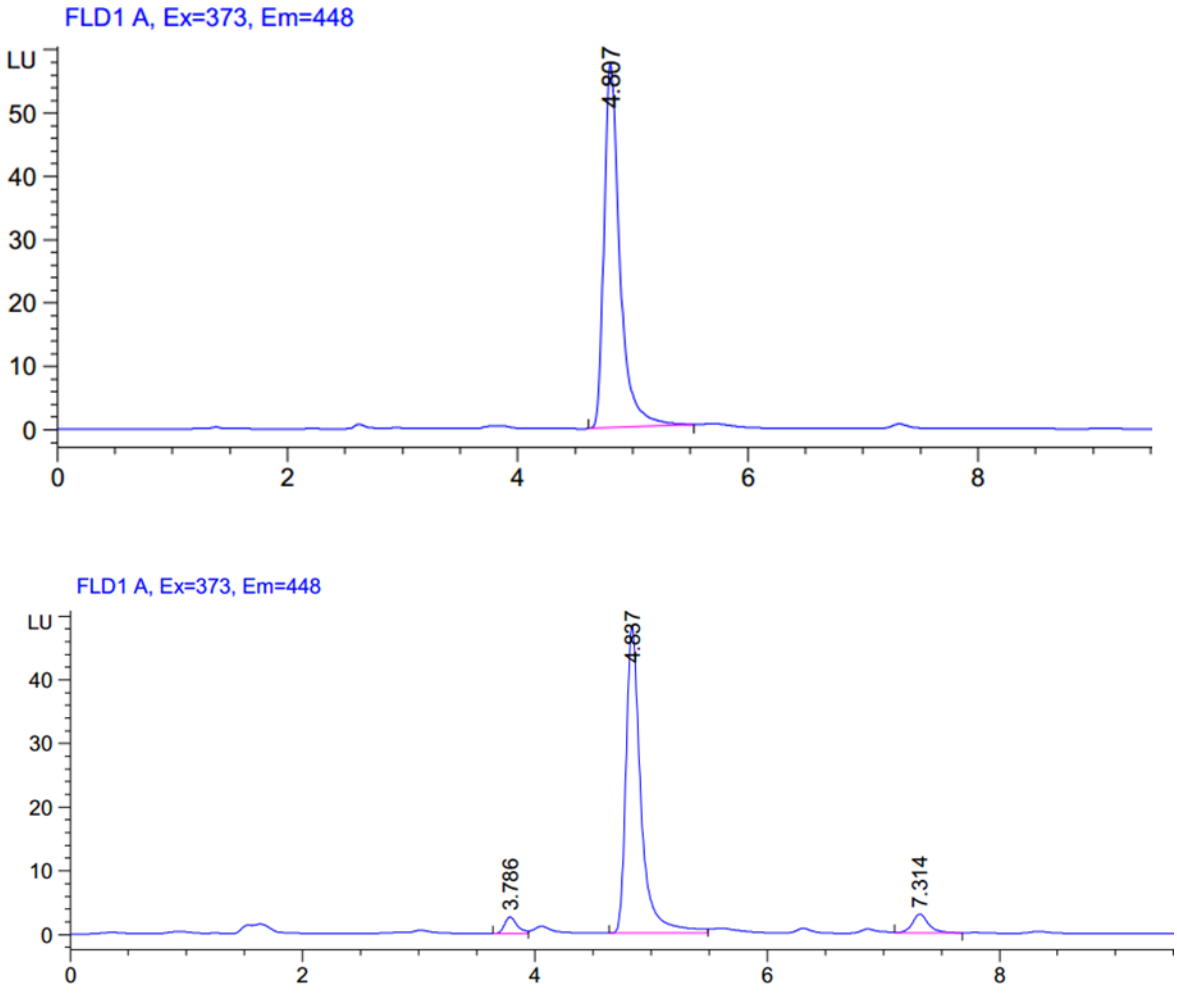


Figure 14. HPLC chromatograms for sialic acid. 50 – 1000 µg of sialic acid standard was serially injected. Only 800µg is shown here as a reference. The peak formed at 4.8 min (top). Sialic acid on recombinant human EPO produced by each ChEPO, ChEPO-S and ChEPO-SG was measured. The sialic acid content of 1 µg of EPO from ChEPO-SG cells is shown here. The major peak also formed at 4.8min, corresponding to the standard (below).

HPLC analysis for recombinant human EPO sialylation was performed several times, and the average sialic acid content on EPO produced by each cell line is shown in Figure 15. Compared to recombinant human EPO purified from ChEPO cell line into which no glycosyltransferases were transfected, EPO sialic acid contents from ChEPO-S and ChEPO-SG cell lines increased ~26% and ~45%, respectively.

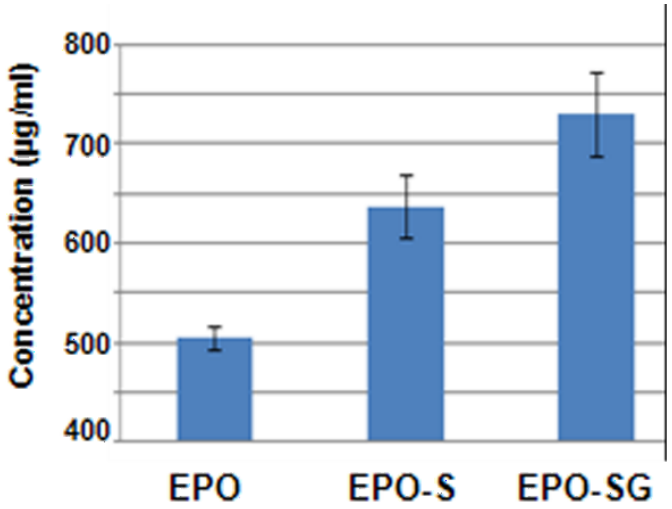


Figure 15. Sialic acid content of recombinant human EPO produced by ChEPO, ChEPO-S and ChEPO-SG cell lines. Sialic acid content of EPO was determined by HPLC with MDB-derivatization method.

Chapter 4: Conclusion

Recombinant biopharmaceutical proteins have revolutionized the treatment of many diseases and have become the most significant and profitable segment of the overall pharmaceutical industry. For these proteins, glycosylation plays an important role in influencing their stability, solubility, pharmacokinetics, bioactivity and immunogenicity [6]. Therefore, to ensure the quality of the recombinant therapeutic glycoproteins, glycoengineering has become an essential part of drug development process for both new molecules and biosimilars [58]. Major strategies to enhance the protein N-glycosylation, especially on the level of terminal sialylation, includes improving the availability and activity of glycosyltransferases, reducing the action of glycosidases, and providing cells more nucleotide sugar substrates [30].

In this study, three glycosyltransferases, ST6GAL1, GNTIV and GNTV were transfected into human EPO producing CHO-K1 cells, creating three cell lines, ChEPO, ChEPO-S and ChEPO-SG. We investigated how these glycosyltransferases influence the level of α2,6-linked sialylation and N-glycan branching at the trimannosyl core of the recombinant human EPO.

Firstly, a stable clone for each ChEPO-S and ChEPO-SG cell line was selected. ChEPO-S was obtained by transfecting ST6GAL1 gene into the ChEPO cell line, creating stable pool by drug selection, and picking the single clone the highest activity with SNA. Similarly, transfecting ChEPO-S with GNTIV and GNTV genes and establishing a stable pool by drug selection gave the ChEPO-SG cells. 23 single clones were randomly chosen and subsequently analyzed by lectin blot with PHA-L. 9 clones with high reaction activity were further evaluated by western blot with anti-GNTIV and

anti-EPO antibodies. Eventually, one clone with both correct GNTIV expression and high EPO production was chosen to be the ChEPO-SG stable line.

The expression and function of ST6GAL1, GNTIV and GNTV in the ChEPO, ChEPO-S and ChEPO-SG stable clone were assessed. RT-PCR analysis confirmed the expression of the mRNA of corresponding glycosyltransferases in cells. Then, western blot verified the expression of glycosyltransferase proteins. To examine the effect of ST6GAL1, GNTIV and GNTV on protein glycosylation, lectin blot and HPLC analysis were carried out. Lectin blot with SNA, PHA-L and DSL for total protein lysate from each cell line indicated that compared to wild-type CHO-K1 and ChEPO cells, ChEPO-S and ChEPO-SG had significantly higher intracellular α2,6-sialylation level, and that ChEPO-SG showed increased β1,4 and β1,6 branching at the trimannosyl core of the N-glycans. Then, recombinant human EPO was purified from each cell line and subjected to lectin blot and HPLC analysis. Both methods proved that EPO from ChEPO-S had a notably higher content of terminal α2,6-sialic acid, compared to that produced by ChEPO cells. Furthermore, EPO purified from ChEPO-SG, with a larger extent of β1,4 and β1,6 branching, exhibited an even higher level of sialylation than ChEPO-S.

This study for the first time investigates and quantifies the effect of simultaneous overexpression of ST6GAL1, GNTIV and GNTV on enhancing the N-glycosylation complexity and terminal sialylation of human EPO produced in CHO cells. This strategy can be utilized to improve the glycosylation of a variety of other recombinant therapeutic glycoproteins in a robust manner. Moreover, our study contributes insights into future glycoengineering of other glycosyltransferases within the N-glycosylation pathway, in

order to expand the complexity and improve sialylation of recombinant proteins of biopharmaceutical and biomedical importance.

Chapter 5: Future Work

5.1 Mass Spectrometry to Analyze Recombinant Human EPO N-glycan Structures

In this study, results from lectin blot and HPLC demonstrated that transfection of ST6GAL1 into recombinant EPO producing CHO cells introduced α2,6-linked sialylation and elevated the overall sialic acid contents. Additional simultaneous transfection of GNTIV and GNTV improved the size and extent of antennarity of the N-glycan, creating more sites available for sialic acid. However, the specific N-glycan structures on recombinant human EPO from each cell line need to be further elucidated by mass spectrometry.

For glycomic analysis, N-glycans need to be cleaved from purified recombinant human EPO as previously described, and then subjected to the mass spectrometer [59]. It will be very beneficial to figure out the number of sialic acid residues per N-glycan as well as the percentages of bi-, tri-, and tetra-branched N-glycan structures of EPO produced by various CHO cell lines, as such we will have a deeper understanding of the impact of ST6GAL1, GNTIV and GNTV on N-glycosylation patterns, and we can better tailor the complex N-glycan structures on therapeutic glycoproteins in future.

5.2 Further Improvement of Sialylation by Nucleotide Sugar Precursor Feeding

As previously mentioned, strategies affecting the level of glycosylation include alteration of the activity of glycosyltransferases for sugar residue transfer, the action of glycosidases on the glycan degradation, and the amount of nucleotide sugar substrate [30]. In this study, three glycosyltransferases, ST6GAL1, GNTIV and GNTV were transfected into CHO cells and therefore increased the protein sialylation level. To further elevate the sialic acid content, future work can be providing CHO cells nucleotide sugar

substrate. Specifically, a sufficient amount of the nucleotide sugar precursor sialic acid and CMP-sialic acid is essential for the sialylation process [60]. However, due to the low cell membrane permeability, it is not helpful to feed CHO cells sialic acid or CMP-sialic acid [61]. Hence, feeding N-acetylmannosamine, ManNAc, the direct precursor for intracellular synthesis of sialic acid with high cell membrane permeability, became an optimal solution (See Figure 4). Gu and Wang fed 20 mM ManNAc to CHO cells expressing recombinant INF- γ, and successfully increased the CMP-sialic acid level by 30 times and protein sialylation level by 15% [61]. Similarly, we will feed ManNAc or its derivatives to ChEPO, ChEPO-S and ChEPO-SG cells, and observe its impact on sialylation level of intracellular proteins and purified recombinant EPO.

5.3 Enhancement of Sialylation by Targeted Gene Silencing

In addition to nucleotide sugar precursor feeding, the sialylation level of recombinant human EPO can be enhanced by treating cells with short interfering RNA (siRNA) and short-hairpin RNA (shRNA) in order to reduce the expression of sialidase genes, as sialidases catalyze the removal of terminal sialic acids from the glycoprotein [62]. Four sialidases (Neu1-4) have been found at different locations in the human, mouse, and rat cells, and their functions varied from each other possibly due to different subcellular locations and substrate specificity [63]. Ngantung et al. used the RNAi technique to decrease the expression of cytosolic sialidase (Neu2) in CHO cells producing recombinant human INF- γ. They found that the sialidase activity was reduced by about 60% and that CHO cells successfully retained the full sialic acid content of the glycoprotein [64]. Similarly, Zhang et al. knocked down the expression of lysosomal sialidase (Neu1) and plasma membrane sialidase (Neu3), and observed an increased

sialylation level by 33% and 26%, respectively. Thus, to further enhance the sialylation of recombinant human EPO produced by ChEPO-S and ChEPO-SG cells, we will knock down the expression of certain sialidases and investigate the change of sialylation level.

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